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Manganese is essential for PlcP metallophosphoesterase activity involved in lipid remodelling in abundant marine heterotrophic bacteria

Running title: Biochemical characterization of PlcP

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Abstract

In vast areas of the ocean, microbes must adapt to the availability of scarce nutrients and a key strategy for reducing the cellular phosphorus (P) quota is to remodel membranes by replacing phospholipids with non-P surrogate lipids. A metallophosphoesterase, PlcP, is essential for lipid remodelling in cosmopolitan marine bacteria of the *Roseobacter* (e.g. *Phaeobacter* sp. MED193) and SAR11 clades (e.g. *Pelagibacter* sp. HTCC7211) and transcription of *plcP* is known to be induced by P limitation. In order to better understand PlcP-mediated lipid remodelling, we sought to characterise PlcP for its metal ion requirement and to determine its selectivity for native bacterial phospholipids. Here, we report the occurrence of a highly conserved binuclear ion centre in PlcPs from MED193 and HTCC7211 and show that manganese is the preferred metal for metallophosphoesterase activity. PlcP displayed high activity towards the major bacterial phospholipids, e.g. phosphatidylglycerol but also phosphatidic acid, a key intermediate in phospholipid biosynthesis. In contrast, phosphatidylserine and phosphatidylinositol, both of which are rare lipids in bacteria, are not preferred substrates. This data suggests that PlcP undertakes a generic lipid remodelling role during the cellular response of marine bacteria to P deficiency and that manganese availability may play a key role in regulating the lipid remodelling process.

Importance

Membrane lipids form the structural basis of all cells. In the marine environment, it is well established that phosphorus availability significantly affects lipid composition in cosmopolitan marine bacteria, whereby non-phosphorus lipids are used to substitute phospholipids in response to phosphorus stress. Central to this lipid remodelling pathway is a newly identified phospholipase C type metallophosphoesterase (PlcP). However, little is known about how

54 PlcP activity is regulated. Here, we determined the role of metal ions in regulating PlcP
55 activity and compared PlcP substrate specificities in PlcP enzymes from two model marine
56 bacteria, the marine *Roseobacter* clade and the SAR11 clade. Our data provides new
57 insights into the regulation of lipid remodelling in these marine bacteria.

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60 Keywords: PlcP, lipid remodelling, *Roseobacter*, SAR11

Introduction

Large expanses of the ocean, particularly surface waters, contain submicromolar concentrations of essential nutrients required for the growth of phytoplankton and heterotrophic bacteria (2, 18), including macronutrients (e.g. P) as well as micronutrients (e.g. iron, manganese). Marine bacteria inhabiting these oligotrophic surface waters have developed sophisticated strategies to meet cellular demands for these essential elements (16). For example, many marine microbes express high affinity membrane transporters e.g. the ABC transporter PstSCAB for phosphate or SitABCD for manganese uptake in order to acquire specific nutrients present at low concentration (9, 24). Other marine microbes such as the diazotrophic marine cyanobacterium *Crocospaera watsonii* can degrade iron-rich metalloproteins to release and recycle iron during limitation (23).

Another important mechanism for adapting to nutrient deficiency is to reduce the cellular requirement for key elements (16). This strategy is now well established in marine phytoplankton and heterotrophic bacteria, whereby membrane phospholipids are replaced by non-P containing surrogate lipids in response to P deficiency (3, 26, 28). In marine phytoplankton, substitution of phospholipids by the sulfur-containing glycolipid, sulfoquinovosyl diacylglycerol (SQDG) significantly reduced the cellular quota for P (28). Our previous work has shown that lipid remodelling is also important in cosmopolitan marine heterotrophic bacteria (26). Members of the marine *Roseobacter* clade and SAR11 clade bacteria can substitute phospholipids, primarily phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) using non-P surrogate lipids such as the betaine lipid diacylglyceryl trimethylhomoserine (DGTS), the glycolipids monoglycosyl diacylglycerol (MGDG) and glucuronic acid diacylglycerol (GADG) and ornithine lipids. Central to this lipid remodelling process in these marine heterotrophic bacteria is a phospholipase C - type phospholipase, designated PlcP, which was first described in the soil bacterium *Sinorhizobium meliloti* (30). It is believed that during lipid remodelling phospholipids are

degraded by PlcP to diacylglycerol (DAG) which then acts as the precursor for the biosynthesis of surrogate lipids in response to P limitation. Indeed, *plcP* deletion mutants no longer synthesize surrogate non-phospholipids, supporting the essential role of PlcP in lipid remodelling (26, 30).

Although PlcP-mediated lipid remodelling appears widespread amongst marine bacteria, since the *plcP* gene has been found in diverse groups of marine heterotrophs including Alphaproteobacteria, Gammaproteobacteria, Flavobacteria and Verrucomicrobia (26), little is known about how PlcP activity is regulated. At the transcriptional level, in the marine bacterium *Phaeobacter* sp. MED193 and the terrestrial bacterium *Sinorhizobium meliloti*, the *plcP* gene is controlled by the two-component system PhoBR, with a Pho box, to which PhoB binds, found upstream of these *plcP* genes (26, 30). The PlcP protein is annotated as a member of the metallophosphoesterase superfamily (Pfam family PF00149, 15). Sequence analyses have shown that PlcP (also known as LpxH2) has moderate sequence similarity to a well-characterized member of PF00149, LpxH, an enzyme catalysing a key step in lipid A biosynthesis in some bacteria (20, 30). Interestingly, recent structural analyses of LpxH have uncovered a conserved binuclear manganese (Mn^{2+}) centre, suggesting that manganese may be important for PlcP activity (5, 19).

In this study, we present a detailed characterization of the PlcP proteins from representatives of the marine *Roseobacter* and SAR11 clades, *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211, respectively, both of which are known to employ PlcP for lipid remodelling in response to P deficiency (3, 26). Specifically, we set out to determine the role of metal ions in regulating PlcP activity and to compare PlcP substrate specificities in PlcP enzymes from these two clades.

Results and discussion

PlcP is a member of the metallophosphoesterase family

Functional domain analysis of PlcP proteins from *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211 revealed the presence of a highly conserved histidine/aspartate/asparagine cage (**Figure 1A**), a signature sequence motif found in the metallophosphoesterase family of proteins (PFam 00149). Subsequent phylogenetic analysis (**Figure 1B**), comprising representative sequences from key members of the metallophosphoesterase family including phosphodiesterases and pyrophosphatases, showed the most closely related protein to PlcP in this family is LpxH, an enzyme catalysing the formation of key intermediates in lipopolysaccharide biosynthesis. PlcP from *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211 has 25% and 22% identity, respectively, to LpxH from *Pseudomonas aeruginosa*. Crystal structures of LpxH from *Pseudomonas aeruginosa* and *Haemophilus influenzae* have been solved recently, both of which showed the presence of a binuclear Mn^{2+} centre in the active site (5, 19).

We then employed homology modelling to predict metal-binding sites in PlcP₁₉₃ (**Figure 2**). Modelling predicted a highly conserved Mn^{2+} coordination centre that was superimposable onto that of the LpxH (PDB 5K8K) structure. Using the PDBeFold server we compared the crystal structure of LpxH to our model. This returned a Q-score of 0.91, with 1 being identical, and a root-mean-square deviation (RMSD, a measure of the average distance between atoms) of 0.25, indicating some small differences between the structures in 222 of the 227 residues compared. With a highly comparable structure, key metal-binding residues of H12, D40, N81, H82, H117, D119, H201 and H203 are shown to be orientated similarly in the two proteins. Likewise, potentially key differences are observed (**Figure 1 and SI video**) in the ligand recognition/binding site between the LpxH crystal structure and the PlcP₁₉₃ model including His134/Asp136, Tyr126/Ala128 and Arg158/Tyr160 respectively, which could play an important role in substrate recognition. Although our modelling procedure is

limited to positioning amino acid residues and not metal-ion co-factors, given the highly similar overlap of amino acid residues around the metal coordination centre we hypothesised that Mn^{2+} metal cofactors would likely be coordinated similarly in PlcP₁₉₃ and so we have shown these ions superimposed as such (**Figure 2B**).

To validate whether Mn^{2+} is indeed required for PlcP activity, we over-expressed and purified the PlcP proteins from *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211 in *E. coli* (**Figure 3A**). The isolated PlcP₁₉₃ and PlcP₇₂₁₁ enzymes from recombinant *E. coli* had no activity. However, when divalent metals were included in the enzyme assay buffer, phosphoesterase activity was immediately restored (**Figure 3B**). Of all the metals tested in this experiment at a range of concentrations (**Figure S1**), Mn^{2+} gives the highest activity, followed by Zn^{2+} and Fe^{3+} . Our data agrees well with known metal requirements for other characterized proteins within this family. For example, a binuclear Mn^{2+} - Mn^{2+} centre is common in Mre11 and LpxH group enzymes, which are evolutionarily more closely related to PlcP than others members of the family (**Figure 1B**). Zn and Fe, on the other hand, have also been found in several proteins of this family, notably GpdQ glycerophosphodiesterase (25). Moreover, our data supports the homology modelling prediction and shows that PlcP from these marine bacteria is a Mn^{2+} -dependent phosphoesterase. Interestingly, both PlcP₁₉₃ and PlcP₇₂₁₁ proteins had phosphodiesterase and phosphomonoesterase activities as assessed using a general substrate containing either a phosphate monoester (PNPP) or a phosphodiester bond (NPCC) (**Figure 3C**).

Site-directed mutants and PlcP activity profile

In order to identify key amino acid residues in the PlcP protein required for phosphoesterase activity, and to further validate the predicted homology model (**Figure 2**), we constructed site-directed mutants of PlcP₁₉₃ and compared their activities with wild type PlcP₁₉₃. The data presented in **Figure 4** confirms the key role of the histidine/aspartate/arginine motifs in PlcP activity. Site-directed mutants of H12, D40/D43, H117/H119, H201/H203 (highlighted in box

1, 2, 4 and 5 respectively in Figure 1A) to alanine almost completely abolished PlcP activity (Figure 4E) supporting our assumption from the homology modelling that these residues are integral to the metal binding site. Similarly, a histidine-to-alanine (H82A, box 3 in Figure 1A) mutation completely abolished PlcP activity. Interestingly, the activity of H82N (53% of wild-type PlcP₁₉₃) and H82R (82% of wild-type PlcP₁₉₃) mutants is largely retained but statistically different to that of the wild type PlcP ($p<0.01$; $p<0.05$, respectively, student *t*-test), suggesting that the presence of a protonated amine group in this position is important in maintaining PlcP activity. The GNXD motif highlighted in box 3 (Figure 1A) includes an interesting sequence variation in several enzymes of this family, including LpxH (arginine, R), YfcE (cysteine, C) and MJ0936 (asparagine, N). Structural determination of the LpxH-substrate complex revealed that the arginine residue is required for binding of the phosphate group of lipid X (19). In YfcE, the residue in this position is thought to be critical for the enzyme to switch between a phosphomonoesterase and a cyclic nucleotide phosphodiesterase (11). Steady-state kinetic measurements of the H82N and H82R mutants of PlcP₁₉₃ showed higher affinity ($K_m=154.8 \pm 4.8 \mu\text{M}$, $182.5 \pm 5.3 \mu\text{M}$, respectively) towards the artificial substrate NPPC compared to the wild-type ($K_m=234.2 \pm 0.6 \mu\text{M}$) PlcP₁₉₃ (Table 1). This enhanced substrate affinity in these mutants may be attributable to the reduced size of the side chain, from an imidazole ring of histidine to an aliphatic side chain of asparagine and arginine (Figure 4B-D).

The substrate profiles of PlcP on phospholipids

Although the use of artificial substrates provides an overview of PlcP phosphodiesterase and phosphomonoesterase activity and how these activities are regulated by metal ions, the native substrates of PlcP are believed to be native bacterial phospholipids in the membrane (3, 26). Phospholipids are degraded by PlcP to generate diacylglycerol (DAG) for the biosynthesis of non-P surrogate lipids in response to P limitation and a *plcP* deletion mutant can no longer synthesise such surrogate lipids (26). In *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211, the native phospholipids are PG and PE. We therefore

hypothesized that PG and PE are the preferred native substrates for PlcP. To test this hypothesis, we used a range of phospholipids in addition to PG and PE. These included phosphatidylcholine (PC), a relative uncommon phospholipid in bacteria and absent in the aforementioned marine bacteria, phosphatidic acid (PA), an intermediate in the biosynthesis of phospholipids, and phosphatidylserine (PS) and phosphatidylinositol (PI) both of which are primarily associated with eukaryotes (14, 27). To rule out any potential effect of fatty acid chain length on PlcP activity across different phospholipids species, all lipid substrates contained sn-1 C16:0/ sn-2 C16:0 palmitic acid. The data presented in **Figure 5** shows that PG is the preferred substrate whereas the enzyme is least active towards PI and PS. Interestingly, PlcP from both marine bacteria showed comparable, if not higher, activity towards PA (containing a phosphate monoester bond), an essential intermediate in phospholipid biosynthesis. Our data therefore suggests that PlcP in these marine bacteria have a relatively broad substrate specificity and are able to divert primary bacterial lipids (or the precursor, PA) through the conversion to DAG for the synthesis of non-P lipids in response to P limitation.

To conclude, our data shows that the activity of purified PlcP from two model marine heterotrophic bacteria is dependent on metal ions particularly manganese, opening up the possibility that manganese availability may play a role in regulating the lipid remodelling process in natural marine systems (**Figure 6**). Dissolved manganese concentrations are in the low nanomolar range (0.1-4 nM) in marine surface waters and it is already known that manganese is important for maintaining optimum photosystem II (PSII) activity in marine phytoplankton (31, 32). Thus, the wide occurrence of ABC-type manganese transporters in SAR11 and marine Roseobacter clades might suggest potential competition with marine phytoplankton for manganese (9). Nevertheless, our observation of PlcP dependence on manganese reiterates more generally the importance of trace metals in regulating the activity of enzymes playing key roles in nutrient transformations in marine systems e.g. zinc in alkaline phosphatase (8) and iron in nitrogenase (23). Moreover, the fact that PlcP appears able to degrade several native bacterial lipids which are common in marine bacteria, e.g. PG

226 and PE, suggests that PlcP undertakes a generic lipid remodelling role among marine
227 heterotrophic bacteria adapted to low P environments.

Experimental procedures

Cloning, expression and purification of PlcP

The wild-type *plcP* from *Pelagibacter* sp. HTCC7211 and the wild type *plcP* and site directed *plcP* mutants (H12A, D40A, D43A, N81A, H82A, H82N, H82R, H117A, D119A, H201A, H203A and D220A) from *Phaeobacter* sp. MED193 were codon-optimized and chemically synthesized by Genscript. The genes were then inserted into the pET28a expression vector using *NdeI* and *BamHI* restriction sites and transformed into *E. coli* BL21(DE3)-CodonPlus-RIL. To induce the expression of PlcP, 2% (v/v) overnight *E. coli* culture grown on lysogeny broth (LB) was inoculated into 600 ml fresh LB broth. Kanamycin was added to LB medium to a final concentration of 50 mg l⁻¹. The cultures were then incubated at 37°C with shaking (200 r.p.m). When the OD₆₀₀ reached ~ 0.5, IPTG was added to a final concentration of 0.2 mM and cells harvested after 4 h at 37°C for PlcP₁₉₃ and its mutants or 8 h at 30°C for PlcP₇₂₁₁. Cells were then harvested by centrifugation and re-suspended in buffer containing 50 mM Tris-HCl, 200 mM NaCl, pH 8.0. Cells were disrupted by sonication. Cell debris was removed by centrifugation at 20,000 ×g for 20 min and the supernatant loaded onto a nickel column (GE healthcare). After washing with buffer containing 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 100 mM imidazole, proteins were eluted with elution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 300 mM imidazole). Purified protein was analysed by SDS-PAGE and protein concentrations were determined using the Bradford assay.

Bioinformatics and homology modelling

The homologous sequence and conserved domain were identified using the BLASTp software provided by the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignment analyses of the deduced amino acid sequences were performed by ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Phylogenetic analysis used the neighbour-joining analysis method using Molecular Evolutionary Genetic Analysis 7.1 software (MEGA, version 7.1) (13). The three-dimensional

model structures of PlcP₁₉₃ and the H82A, H82N and H82R mutants were generated by submitting the respective amino acid sequences to the Phyre 2 protein modelling and structure prediction server (10) and selecting the overall best scoring model in terms of coverage and confidence. All protein structure models were visualised in Chimera (22).

PlcP activity assay using artificial substrates

PlcP activity was measured in 96 well microplates using 1 mM *p*-nitrophenylphosphorylcholine (NPPC) or *p*-nitrophenylphosphate (PNPP) as a substrate, containing 1 µM purified enzyme in 50 mM Tris-HCl (pH 9.5), 60% (w/v) sorbitol, 1 mM MnCl₂. Enzyme activity was measured at 65 °C for 30 min and absorbance was monitored at 405 nm for the formation of *p*-nitrophenol. One unit of phospholipase activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol per min under the standard conditions. K_m and V_{max} values were calculated using Hanes-Wolff plots at varying concentrations of substrates (0.02-1.0 mM) in three replicates.

PlcP activity assay using phospholipids

A range of phospholipids were used to test PlcP specificity, including 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (PG), 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE), 1, 2-dipalmitoyl-sn-glycero-3-phosphate sodium salt (PA), 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine sodium salt (PS) and 1, 2-dipalmitoyl-sn-glycero-3-phospho-1'-myo-inositol ammonium salt (PI) (7). The standard reaction mixture (300 µl) contained 2.5 µM purified enzyme in 50 mM Tris-HCl (pH 9.5), 1 mM MnCl₂ and phospholipids (0.1-0.8 mM), which was incubated for 30 min at 65 °C. Phospholipids and the common degradation product, diacylglycerol (DAG) were extracted according to the Folch method using methanol:chloroform:water at a ratio of 1:2:0.6 (v/v/v). The lipid extract was dried under nitrogen gas at room temperature. The dried lipids were re-suspended in acetonitrile and ammonium acetate (10 mM, pH 9.2) at a ratio of 95:5 (v/v), and analysed by liquid chromatography-mass spectrometry (LC-MS).

Phospholipid characterization and quantification by LC-MS

Phospholipids and PlcP-hydrolysed lipid products were analysed by LC-MS using a Dionex UltiMate 3000 LC system (Thermo Scientific, Walham, MA) coupled to a Bruker amazon SL electrospray- ion trap mass spectrometer (Billerica, MA). A BEH Amide XP column (2.5 μ m, 3 mm \times 150 mm) was obtained from Waters (Milford, MA) and used for the chromatographic separation using a mobile phase consisting of acetonitrile (solvent A) and 10 mM ammonium acetate, pH 9.2 (solvent B). The column was equilibrated for 10 min with 95:5 (v/v) A:B prior to sample injection. The separation of phospholipids was conducted using a stepwise gradient starting from 95% (v/v) A: 5% (v/v) B to 70% (v/v) A: 30% (v/v) B after 15 min with a constant flow rate of 150 μ l min⁻¹. Instrument settings for the positive ion ESI/MS and MS/MS analysis of phospholipids were as follows: capillary voltage of 4500 V; end plate offset of 500 V; 8 L min⁻¹ drying gas at 250 °C; nebulizing gas pressure of 15 psi. Data analysis was performed using the Compass DataAnalysis and QuantAnalysis 4.2 software (Bruker, Billerica, MA). PlcP activity towards phospholipids was measured by quantifying 1,2-dipalmitoyl-sn-glycerol (DAG) formation. A standard calibration curve for DAG was generated by correlating peak area to DAG quantity. A concentrated stock solution of DAG was prepared by dissolving in chloroform at a concentration of 0.5 mg ml⁻¹. The concentrated stock was further diluted in chloroform to generate standards at 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mg ml⁻¹.

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References

1. Andreeva, A.V., and Kutuzov, M.A. (2004) Widespread presence of “bacterial-like” PPP phosphatases in eukaryotes. *BMC Evol. Biol.* 4: 47.
2. Bristow, L.A., Mohr, W., Ahmerkamp, S., and Kuypers, M.M.M. (2017) Nutrients that limit growth in the ocean. *Curr Biol.* 27:R474-R478.
3. Carini, P., Van Mooy, B.A., Thrash, J.C., White, A., Zhao, Y., Campbell, E.O., Fredricks, H.F., Giovannoni, S.J. (2015) SAR11 lipid renovation in response to phosphate starvation. *Proc Natl Acad Sci USA.* 112: 7767-7772.
4. Chen, S., Yakunin, A.F., Kuznetsova, E., Busso, D., Pufan, R., Proudfoot, M., Kim, R., Kim, S.H. (2004) Structural and functional characterization of a novel phosphodiesterase from *Methanococcus jannaschii*. *J Biol Chem* 279: 31854-31862.
5. Cho, J., Lee, C.J., Zhao, J., Young, H.E., Zhou, P. (2016) Structure of the essential *Haemophilus influenzae* UDP-diacylglycerol pyrophosphohydrolase LpxH in lipid A biosynthesis. *Nat Microbiol.* 1: 16154. doi: 10.1038/nmicrobiol.2016.154.
6. Flores-Diaz, M., Monturiol-Gross, L., Naylor, C., Alape-Giron, A., Flieger, A. (2016) Bacterial sphingomyelinases and phospholipases as virulence factors. *Microbiol Mol Biol Rev* 80: 597-628.
7. Folch, J., Lees, M., Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem.* 226: 497-509.

8. Gudjónsdóttir, K., and Asgeirsson, B. (2008) Effects of replacing active site residues in a cold-active alkaline phosphatase with those found in its mesophilic counterpart from *Escherichia coli*. *FEBS J.* 275:117-127.
9. Green, R.T., Todd, J.D., Johnston, A.W. (2013) Manganese uptake in marine bacteria; the novel MntX transporter is widespread in *Roseobacters*, *Vibrios*, *Alteromonadales* and the SAR11 and SAR116 clades. *ISME J.* 7: 581-591.
10. Kelly, L., Mezulis, S., Yates, C., Wass, M., and Sternburg, M. (2015) The Phyre2 web portal for protein modelling, prediction and analyses. *Nature Protocol* 10: 854-858.
11. Keppetipola, N., and Shuman, S. (2008) A phosphate-binding histidine of binuclear metallophosphodiesterase enzyme is a determinant of 2'3'-cyclic nucleotide phosphodiesterase activity. *J Biol Chem.* 283: 30942-30949.
12. Khalid, M.F., Damha, M.J., Shuman, S., Schwer, B. (2005) Structure-function analyses of yeast RNA debranching enzyme (Dbr1), a manganese-dependent phosphodiesterase. *Nucleic Acids Res* 33: 6349-6360.
13. Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 33: 1870-1874.
14. Lopez-Lara, I.M., and Geiger, O. (2017) Bacterial lipid diversity. *Biochim Biophys Acta* 1862: 1287-1299.
15. Matage, N., Podobik, M., Visweswariah, S. (2015) Metallophosphoesterases: structural fidelity with functional promiscuity. *Biochem J.* 467: 201-216.

16. Merchant, S.S., Helmann, J.D. (2012) Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. *Adv Microb Physiol.* 60: 91-210.
17. Miller, D.J., Shuvalova, L., Evdokimova, E., Savchenko, A., Yakunin, A.F. and Anderson, W.F. (2007) Structural and biochemical characterization of a novel Mn²⁺-dependent phosphodiesterase encoded by the *yfcE* gene. *Protein Sci.* 16: 1338–1348.
18. Moore, C.M., Mills, M.M., Arrigo, K.R., Berman-Frank, I., Bopp, L., Boyd, P.W., et al. (2013) Processes and patterns of oceanic nutrient limitation. *Nat Geosci* 6: 701–710.
19. Okada, C., Wakabayashi, H., Kobayashi, M., Shinoda, A., Tanaka, I., Yao, M. (2016) Crystal structures of the UDP-diacylglycosamine pyrophosphohydrolase LpxH from *Pseudomonas aeruginosa*. *Sci Rep.* 6: 32822.
20. Opiyo, S.O., Pardy, R.L., Moriyama, H., Moriyama, E.N. (2010) Evolution of the Kdo2-lipid A biosynthesis in bacteria. *BMC Evol Biol.* 10: 362.
21. Paull, T.T., Gellert, M. (1998) The 3' to 5' exonuclease activity of Mre 11 facilitates repair DNA of double-strand breaks. *Mol Cell* 1: 969-979.
22. Pettersen, E., Goddard, T., Huang, C., Couch, G., Greenblatt, D., Meng, E. and Ferrin T. (2004) UCSF Chimera - A visualization system for exploratory research and analysis. *J Comput Chem* 25: 1605-1612.
23. Saito, M.A., Bertrand, E.M., Dutkiewicz, S., Bulygin, V.V., Moran, D.M., Monteiro, F.M., Follows, M.J., Valois, F.W., Waterbury, J.B. (2011) Iron conservation by

reduction of metalloenzyme inventories in the marine diazotroph *Crocospaera watsonii*. *Proc Natl Acad Sci USA*. 108: 2184-2189.

24. Scanlan, D.J., Ostrowski, M., Mazard, S., Dufresne, A., Garczarek, L., Hess, W.R., Post, A.F., Hagemann, M., Paulsen, I., Partensky, F. (2009) Ecological genomics of marine picocyanobacteria. *Microbiol Mol Biol Rev*. 73: 249-299.

25. Schenk, G., Mitić, N., Gahan, L.R., Ollis, D.L., McGeary, R.P., Guddat, L.W. (2012) Binuclear metallohydrolases: complex mechanistic strategies for a simple chemical reaction. *Acc Chem Res*. 45: 1593-603.

26. Sebastián, M., Smith, A.F., González, J.M., Fredricks, H.F., Van Mooy, B., Koblížek, M., Brandsma, J., Koster, G., Mestre, M., Mostajir, B., Pitta, P., Postle, A.D., Sánchez, P., Gasol, J.M., Scanlan, D.J., Chen Y. (2016) Lipid remodelling is a widespread strategy in marine heterotrophic bacteria upon phosphorus deficiency. *ISME J* 10: 968-978.

27. Sohlenkamp, C., Lopez-Lara, I.M., Geiger, O. (2003) Biosynthesis of phosphatidylcholine in bacteria. *Prog Lipid Res* 42: 115-162.

28. Van Mooy, B.A., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblížek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappé, M.S., Webb, E.A. (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature* 458: 69-72.

29. Wendel, B.M., Cole, J.M., Courcelle, C.T., Courcelle, J. (2018) SbcC-SbcD and ExoI process convergent forks to complete chromosome replication. *Proc Natl Acad Sci USA*. 115: 349-354. doi: 10.1073/pnas.1715960114.

420

421 30. Zavaleta-Pastor, M., Sohlenkamp, C., Gao, J.L., Guan, Z., Zaheer, R., Finan, T.M.,
422 Raetz, C.R., López-Lara, I.M., Geiger, O. (2010) *Sinorhizobium meliloti*
423 phospholipase C required for lipid remodelling during phosphorus limitation. *Proc*
424 *Natl Acad Sci USA*. 107: 302-307.

425

426 31. Salomon, E., and Keren, N. (2011) Manganese limitation induces changes in the
427 activity and in the organization of photosynthetic complexes in the cyanobacterium
428 *Synechocystis* sp. strain PCC 6803. *Plant Physiol*. 155:571-579.

429

430 32. van Hulst, M., Middag, R., Dutay J-C., de Barr, H., Roy-Barman, M., Gehlen, M., et
431 al., (2017) Manganese in the west Atlantic Ocean in the context of the first global
432 ocean circulation model of manganese. *Biogeosci*. 14:1123-1152.

Table 1 Kinetic parameters of PlcP₇₂₁₁ wild type, PlcP₁₉₃ wild type, and PlcP₁₉₃ mutant enzymes following the hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC)^a.

Protein	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
PlcP ₇₂₁₁	41.8 ± 6.3	275.6 ± 5.7	151.7 ± 5.9
PlcP ₁₉₃	38.6 ± 5.2	234.2 ± 6.3	164.8 ± 5.6
H82R	25.5 ± 3.8	182.5 ± 5.3	140.0 ± 3.9
H82N	18.6 ± 4.2	154.8 ± 4.8	120.1 ± 4.5
H82A	0.12 ± 0.1	17.2 ± 2.3	7.0 ± 2.5

^a Values are mean ± standard deviation of three independent experiments.

Figure legends

Figure 1 Multiple sequence alignment and functional domain analyses of PlcP proteins. PLC₁₉₃, PlcP of *Phaeobacter* sp. MED193; PLC₇₂₁₁, PlcP of *Pelagibacter* sp. HTCC7211; PlcP_{Sm}, PlcP of *Sinorhizobium meliloti* (30).

A) Multiple sequence alignment of PlcP and closely related LpxH enzymes. The 6 conserved motifs are highlighted in grey. The conserved histidine residue in PlcP (H82) is highlighted in green.

B) Neighbour-joining phylogenetic analysis between members of the metallophosphoesterase family (PFam 00149) including proteins closely related to PlcP: PaLpxH, LpxH from *Pseudomonas aeruginosa* (19); HiLpx, LpxH from *Haemophilus influenza* (5). LpxH displays pyrophosphatase activity and acts on UDP-2, 3-diacylglucosamine to produce lipid X, a key precursor for the formation of lipid A in lipopolysaccharide biosynthesis. More distantly related members of the metallophosphoesterase family include: MJ0936, which represents a group of novel phosphodiesterases which do not degrade phosphomonoesters (4); Mre11/SbcD which are bacterial and archaeal DNA phosphodiesterases involved in DNA repair (21, 29); Dbr1, which is a group of phosphodiester nucleases that act on RNA (12); CpdA, CpdB and cAMP phosphodiesterases which are cyclic nucleotide phosphodiesterases; ApaH, which represents a group of enzymes with pyrophosphatase and protein phosphatase activities (1); YfcE, which represents a group of small metallophosphoesterases showing phosphodiesterase activity (17); Sphingomyelinase, which is a group of hydrolases responsible for breaking down sphingomyelin to phosphocholine and ceramide (6). Numbers indicate bootstrap values (only values >50 are shown).

Figure 2 Homology modelling showing the predicted structure of PlcP₁₉₃ and the metal-binding pocket. The signature arginine residue in LpxH (Arg81) is substituted by a histidine residue in PlcP (His82).

Figure 3 PlcP displays manganese-dependent phosphomonoesterase and phosphodiesterase activities.

A) Over-expression and purification of PlcP from *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211. M, protein molecular weight marker. Lane 1, cell-free supernatant induced with Isopropyl β -D-1-thiogalactopyranoside (IPTG); lane 2, cell-free supernatant without IPTG induction; lane 3, purified PlcP protein (molecular weight estimated to be ~ 27 kDa).

B) PlcP activity assays in the presence of various divalent metal ions (1 mM). Values are mean \pm standard deviation of three replicated measurements.

C) PlcP activity assays using *p*-nitrophenylphosphorylcholine (NPPC) or *p*-nitrophenylphosphate (PNPP). Values are mean \pm standard deviation of three replicated measurements.

Figure 4 Homology modelling prediction of the metal-coordination centre in the PlcP₁₉₃ enzyme (**A**) and the site-directed mutants of His82 to Ala82 (**B**), Arg82 (**C**) and Asn82 (**D**). Specific activities of site directed mutants of PlcP₁₉₃ are measured using *p*-nitrophenylphosphorylcholine (NPPC) as the substrate (**E**). Values are mean \pm standard deviation of three replicated measurements.

Figure 5 Relative activity of the degradation of phospholipids by PlcP₁₉₃ and PlcP₇₂₁₁. Activity was measured by quantifying the formation of the common product diacylglycerol (DAG) in these reactions. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PS,

phosphatidylserine. Values are mean \pm standard deviation of three replicated measurements.

Figure 6 A schematic overview of the PlcP-mediated lipid remodelling pathway and its regulation in representative marine bacteria. The major lipids in *Phaeobacter* sp. MED193 and *Pelagibacter* sp. HTCC7211 during P replete conditions are two phospholipids (highlighted in grey), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). During P stress, the two component signal transduction system PhoBR is activated and the phosphorylated PhoB activates not only the expression of the high affinity ABC transporter for phosphate (PstSABC) but also the transcription of the *plcP* gene. A conserved phoB binding site in the *plcP* promoter has previously been identified in these bacteria (26). The purified PlcP protein requires manganese for activity (Figure 3). Manganese is likely transported into the cell through either the SitABCD or the MntX transporter systems that are present in these marine heterotrophic bacteria (9). Active PlcP can convert PE, PG or its biosynthesis precursor phosphatidic acid (PA) to diacylglycerol (DAG), which serves as the building block for the biosynthesis of alternative P-free surrogate lipids (highlighted in blue), including diacylglycerol trimethylhomoserine (DGTS) and the glycolipids monoglycosyl diacylglycerol (MGDG) and glucuronic acid diacylglycerol (GADG) (3, 26).